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DAF marker tightly linked to a major locus for *Ascochyta* blight resistance in chickpea (*Cicer arietinum L.*)

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Summary

Resistance of chickpea against the disease caused by the ascomycete *Ascochyta rabiei* is encoded by two or three quantitative trait loci, QTL1, QTL2 and QTL 3. A total of 94 recombinant inbred lines developed from a wide cross between a resistant chickpea line and a susceptible accession of *Cicer reticulatum*, a close relative of cultivated chickpea, was used to identify markers closely linked to QTL1 by DNA amplification fingerprinting in combination with bulked segregant analysis. Of 312 random 10mer oligonucleotides, 3 produced five polymorphic bands between the parents and bulks. Two of them were transferred to the population on which the recent genetic map of chickpea is based, and mapped to linkage group 4. These markers, OPS06-1 and OPS03-1, were linked at LOD-scores above 5 to markers UBC733B and UBC181A flanking the major ascochyta resistance locus. OPS06-1 mapped at the peak of the QTL between markers UBC733B (distance 4.1 cM) and UBC181A (distance 9.6 cM), while OPS03-1 mapped 25.1 cM away from marker UBC733B on the other flank of the resistance locus. STMS markers localised on this linkage group were transferred to the population segregating for ascochyta resistance. Three of these markers were closely linked to QTL1. Twelve of 14 STMS markers could be used in both populations. The order of STMS markers was essentially similar in both populations, with differences in map distances between them. The availability of flanking STMS markers for the major resistance locus QTL1 will help to elucidate the complex resistance against different *Ascochyta* pathotypes in future.

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important grain legume crop in the world. It is grown in the Indian subcontinent, West Asia, North Africa (WANA), the Mediterranean basin, the Americas and Australia. Until now, conventional breeding has increased chickpea yields at a discouragingly low rate of 0.6% annually. Chronic cycles of ascochyta blight, caused by the ascomycete *Ascochyta rabiei* (Pass) Labr. are major constraints to yield improvement in northern regions of the Indian subcontinent and the WANA region. The fungus attacks all aerial parts of the plant, thereby causing yield losses from 10 to 100% under conditions that also favor chickpea growth (Nene & Sheila, 1992). To overcome these constraints, high yielding cultivars with resistance to soilborne and foliar diseases and tolerance to abiotic stresses such as cold and drought are required (Nene & Sheila, 1992; Saxena, 1992). Despite considerable international efforts to breed such lines conventionally, cultivars with long-lasting resistance against the fungus are still lacking. One of the problems en-

countered by breeders is the divergence of the pathogen. For example, six races of the fungus have been identified in Syria and Lebanon (Reddy & Kabbabeh, 1985), and 12 pathotypes have been reported from northern India (Singh, 1985). Gowen (1983) found an isolate that was lethal to formerly resistant cultivars indicating that new pathotypes emerge rapidly. The situation becomes even more complicated, because the fungus can also occur in its sexual state *Didymella* (formerly *Mycosphaerella*) *rabiei* (Kov.) V. Arx (review in Kaiser, 1992). Sexual recombination increases the probability that new pathotypes will appear.

The second problem for breeding resistant cultivars is the complexity of resistance. The picture emerging from different studies is confusing. Depending on the isolate of the fungus and the cultivars tested, either one dominant, one recessive and one dominant, or one recessive resistance genes were reported. Also, two complementary recessive, and two complementary dominant genes were detected (Nene & Sheila, 1992; Singh et al., 1992). To complicate the picture even more, additional genes were reported to modify the expression of resistance (Muehlbauer & Singh, 1987; Santra et al., 2000; Tekeoglu et al., 2000). It is not clear, whether the reported genes represent the same or different loci because allelic tests have not been performed.

Another reason for the apparent confusion is that in earlier studies different methods were used to estimate disease severity, and inheritance of resistance was studied in F_2 or backcross populations grown for a single year in only one location. Only recently, Tekeoglu et al. (2000) used three different populations of recombinant inbred lines (RILs, Burr et al., 1988) derived from one inter-specific cross between *C. arietinum* FLIP 84-92C (resistant parent) and *C. reticulatum* PI 599072 (susceptible parent), and two intra-species crosses of resistant and susceptible accessions of *C. arietinum* to score segregation of resistance in the field for three consecutive years. This study revealed that three major recessive and complementary genes together with several modifiers confer resistance against the fungus. Absence of one or two of the major genes led to susceptibility, whereas the modifiers determined the degree of resistance (Tekeoglu et al., 2000).

The RILs of the interspecific cross were used to develop a genetic map of chickpea (Santra et al., 2000) with random amplified polymorphic DNA (RAPD, Williams et al., 1990), inter-simple-sequence-repeat (ISSR, Gupta et al., 1994) and isozyme markers. The disease scores of the individual RILs were used for an analysis of quantitative trait loci (QTLs, Young, 1996). Interval mapping revealed the two major QTL-1 and QTL-2, with LOD scores of 17.2 and 7.3, respectively, located on linkage group 6 and 1 of the maps of Gaur and Slinkard (1990) and Kazan et al. (1993), respectively, that individually accounted for 42.5% (41.4%) and 19.9% (17.2%) of the resistance against *A. rabiei* in 1997 and 1998, respectively. Two RAPD markers, UBC733b and UBC181a, flanked QTL-1 and mapped 10.9 cM from each other, whereas QTL-2 was flanked by an ISSR and an isozyme marker that were 5.9 cM apart. The two major QTLs were detected using resistance scoring data from two subsequent years. However, a third QTL (3) showed up in the data from only one year, and was localized on linkage group 4 at a LOD score of 3.04 – slightly above the threshold of acceptance – and flanked on either side by a RAPD and an ISSR marker that were 11.7 cM apart. The two major genes together accounted for 50.3 and 45.0%, respectively, of the total estimated phenotypic variation in two subsequent years (Santra et al., 2000).

As in chickpea, resistances in lentil and pea against ascochyta blight diseases caused by *Ascochyta lentis* and *A. pisi*, respectively, are controlled by more than one gene (polygenic trait). However, resistance in chickpea resembles that in pea, where 3 genes account for 71% of the variation in resistance (Dirlewanger et al., 1994), while in lentil a single locus accounted for almost 90% of the phenotypic variation between resistant and susceptible individuals of an F_3 population (Ford et al., 1999).

Recently, an STMS marker map has been developed for chickpea (Hüttel et al., 1999; Winter et al., 1999). This map allows determination of the positions of loci, defined in any population, on the recent genomic map of chickpea (Winter et al., 2000) by simply applying STMS markers with known map positions in the population segregating for the trait of interest. However, the validity of these maps and the transfer of STMS markers between different segregating populations has not yet been proven for chickpea.

Building on the work of both Tekeoglu et al. (2000), Santra et al. (2000) and Winter et al. (1999, 2000) the present study aimed at (1) detecting STMS and other markers closely linked to major resistance loci against *A. rabiei*, (2) localizing these loci on the most advanced genetic map of chickpea and evaluating the order of markers on this map, and (3) demonstrating transferability of STMS markers. Further, we tested whether the oftenly doubted application

of bulked segregant analysis (BSA, Michelmore et al., 1991) for mapping of QTLs was applicable to loci accounting for less than 50% of the phenotypic variation in segregating populations.

Materials and methods

Plant material and DNA extraction

The first population of RILs used here was derived from a wide cross between ICC4958 and *C. reticulatum* accession PI 489777. This population has been exploited to generate the most extended map of the chickpea genome (Winter et al., 2000) and is designated as wide cross 1 (WC1). Plants were propagated in the greenhouse, and DNA was isolated from young leaflets by a modified cetyl-trimethyl ammoniumbromide (CTAB) protocol (Weising et al., 1995). Contaminating polysaccharides were selectively precipitated (Michaels et al., 1994). The second mapping population of F_7:$_8$ recombinant inbred lines (RILs) obtained from a cross between chickpea accession FLIP 84-92C (ascochyta blight resistant parent) and *C. reticulatum* accession PI 599072 (susceptible parent), and scoring for resistance to ascochyta blight in parents and RILs has been described (Tekeoglu et al., 2000; Santra et al., 2000). We refer to this population as wide cross 2 (WC2).

DNA amplification fingerprinting and electrophoresis

PCR was carried out on a Perkin Elmer Geneamp 9700 thermal cycler using random 8mers or 10mers procured from Roth (Germany), Operon (USA), or Eurogentec (Belgium) as primers. Each 15 μl PCR reaction contained 1.5 μl 10 $\times$ PCR buffer (Eurogentec), 10 mM of each dNTP (MBI Fermentas, Estonia), 0.4 U 'Silverstar' DNA polymerase (Eurogentec), 40 pmol oligonucleotide primer, and 1 ng/μl template DNA (Caetano-Anolles et al., 1991). The DNA was first denatured for 2 min at 95 °C, followed by 40 cycles of 1.5 min denaturation at 95 °C, 1 min annealing at 35 °C and 2 min elongation at 72 °C, with a final elongation of 2 min at the same temperature. The reaction products were separated on 1.8% agarose or 6% polyacrylamide gels, and visualized with ethidium bromide fluorescence, or silver staining.

STMS marker technology

STMS markers and their positions on the genetic chickpea map have been described (Hüttel et al., 1999, Winter et al., 1999, 2000).

Bulked segregant analysis (BSA) and selective genotyping

BSA was performed essentially as defined by Michelmore et al. (1991). Individual RILs that displayed consistant disease severity ratings of 1 to 3 over two subsequent years in the study of Tekeoglu et al. (2000) were grouped as resistant class, and lines with scores of 9 were grouped as susceptible class. Twelve each from resistant and susceptible lines represented the resistant and susceptible bulks. For BSA, 1.0 ng of DNA of each of 12 individuals from the resistant or susceptible bulk, respectively, were mixed and used as template.

Primers giving rise to polymorphic bands between the bulks were further tested for possible linkage to the resistance locus by selective genotyping of 7 resistant and 7 susceptible RILs. Here, DAF was performed on 15 ng of DNA of each individual RIL and the 2 parental lines. Bands present in all individuals of one group, but absent in all individuals of the other were assumed to be linked to one or the other resistance locus, and the respective primers were tested on the whole population.

Linkage analysis

Marker order was determined using Mapmaker V3.0 (Lander et al., 1987). Markers were placed on the map relative to those already reported (Santra et al., 1998, Winter et al., 1999, 2000), employing the '*try*' routine of the program. Final marker order was confirmed by multipoint analysis provided by Mapmakers '*ripple*' function, and map distances were calculated with Mapmakers 'Kosambi' function.

Development of a single-locus marker from a linked DAF band

A 430 bp amplicon from primer OPS-06-1 tightly linked to the major resistance locus was excised from the gel, the DNA extracted with QIAquick (Qiagen), ligated into pGEM-T-easy vector (Promega) and electroporated into *E. coli* DH10B electrocompetent cells using a Biorad Genepulser. The identity of inserts

from recombinant plasmids was proven by hybridization of radiolabelled inserts to the bands of each parental line and 7 from each susceptible and resistant RILs. Inserts that detected the correct linked bands were sequenced, and nested primers designed as described by Hüttel et al. (1999). Primers were used to amplify a single band from chickpea DNA in a total PCR reaction volume of 25 μl, containing 20 ng DNA, 1.5 mM $MgCl_2$, 250 μm dNTPs, 2μM primer and 1 U 'Silverstar' *Taq* DNA polymerase (Eurogentec).

Results

A total of 312 DAF primers were screened for polymorphisms between the bulks. Of these, 299 generated a reproducible banding profile. Primers detecting polymorphism between the bulks were used to screen the parents and seven individual lines from each of the bulks. This procedure identified bands inherited from either FLIP 84-92C or PI 489777. Three primers produced five polymorphic bands between the bulks as well as the parents (Figure 1). These were used to screen the population. All of them segregated according to Mendelian rules as dominant loci and none showed distorted segregation. As shown in Figure 2, two of these markers, OPS06-1 and OPS03-1 were linked at LOD scores above 5 to markers UBC733B and UBC181A flanking the major ascochyta resistance locus (Santra et al., 2000). Marker OPS06-1 was 4.1 cM away from marker UBC733B, while marker OPS03-1 mapped 25.1 cM away from this marker. A third DAF marker, OPK06-5, was located on the distal end of the linkage group (LG) about 30 cM away from the locus (not shown in Figure 2).

Marker OPS03-1 could be transferred to WC1, on which the advanced genomic map of chickpea is based (Winter et al., 1999, 2000). The transfer was possible, because it showed the same polymorphism as in WC2, in which it was tightly linked to STMS markers STMS 11, GA24 and GAA47 on LG 4 (Winter et al., 2000). Therefore, the major *Ascochyta* resistance locus could be localized on this linkage group. To confirm the results obtained with the DAF marker, and to evaluate the marker order on this linkage group in another cross, these STMS markers were tested on WC2. The highly polymorphic nature of microsatellites in the chickpea genome made it possible to use all three STMS for linkage analysis in WC2. They were linked to OPS03-1, UBC733B, OPS06-1, UBC181A and to the resistance locus at LOD scores of 5 and above. STMS marker GAA47 was located only 21.5 cM away from marker UBC733B, and is presently the most closely linked codominant marker for this locus.

Since another codominant marker on the other flank of the resistance locus would be advantageous for breeding purposes, we tried to generate a single-locus, sequence characterized region (SCAR) marker from OPS06-1. The corresponding band from one parent was cloned and sequenced, and nested primers complementary to the ends of the fragment designed (primer sequences SR2L: GGAGAGCATGGAGACTCAAAAA; SR2R: CGGCTAAACCTAGTCGTTCAAA). Unfortunately, this primer pair gave rise to unique bands of similar size and sequence in both parental lines, and thus the marker could not be mapped in both populations.

To evaluate the order of STMS markers on LG 4 of the current genetic map of chickpea (Figure 2), the additional 11 STMS located on this LG were also tested on WC2. Of these, 9 could be mapped in WC2. Markers STMS 26 and TAA130 did not show polymorphism between the parental lines, and were not mapped. The marker order was essentially the same in both populations (Figure 2). Only orders of closely linked markers were changed (for example, OPS03-1 and marker doublet STMS11 / GA24, between which no recombination was detected in both crosses, as well as TA13 / TA20 and TAA46 / STMS24).

Though the order of markers did not change significantly between the two maps, the distances between the markers in some regions was lower in the WC2 map. For example, a cluster of 4 STMS markers (TR20, TA146, TA72 and TA2) did not show recombination in WC2, whereas the distances between them in WC1 ranged from 0.7 and 2.3 cM. Also, distances between more distantly coupled markers are sometimes larger in WC1. For example, map distances between markers TAA46 and GA2 summed up to 177 cM in WC1 as compared to only 136 cM in WC2. However, this is not true for the distance between markers GA2 and GAA47, which is 25 cM in WC1, but 63 cM in WC2.

Discussion

Ascochyta blight is a severe disease of several legumes including chickpea, field pea and lentil. At least in chickpea and lentil there are conflicting reports about the mechanism of resistance. The genetic basis for resistance to *A. lentis* was recently clarified as single

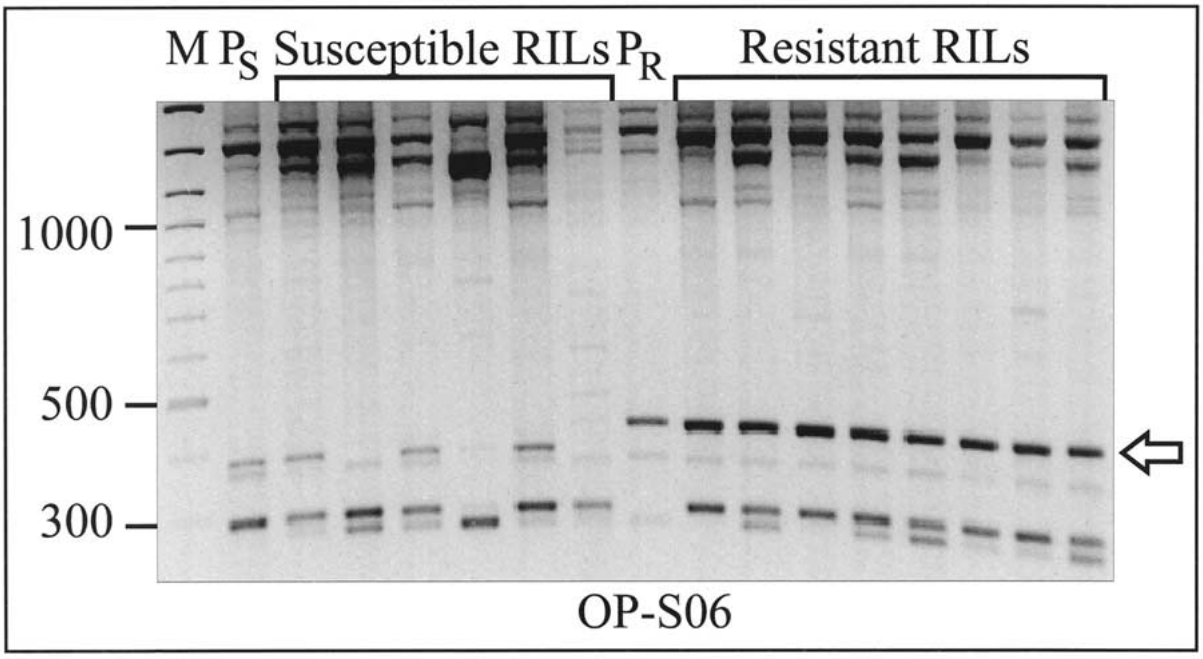

Figure 1. Segregation of marker band OP-S06-1 (indicated by arrow) in *Ascochyta* susceptible and resistant RILs, respectively. Molecular weight size standards are given in base pairs (bp). P_S, P_R: susceptible and resistant parent, respectively.

dominant locus, which explained more than 90% of the variance between resistant and susceptible lentil lines (Ford et al., 1999). These authors used RAPD analysis to generate a small genetic map with linked markers at distances of 6 and 14 cM flanking the resistance locus. They also demonstrated that BSA can be used to tag at least the major resistance locus with molecular markers, though ascochyta resistance is not completely monogenic. Here we applied a similar approach to identify DAF markers linked to the locus contributing most to resistance against *A. rabiei* in chickpea (i.e. QTL-1; Santra et al., 2000). Though this locus explains less than 50% of the resistance reaction, the approach was successful. Three out of 5 markers that were polymorphic between the bulks, parents and also in the segregants (selective genotyping) were linked to this resistance locus. BSA then is an efficient way of identifying closely linked markers for incompletely monogenic ascochyta resistances, and possibly also for other major QTLs. However, since at least two loci are necessary to explain about 50% of the resistance (namely QTL-1 and QTL-2), all individuals of the resistant bulk should possess both loci. Therefore, it was potentially possible to tag both loci with this approach. The fact, that we tagged only one of these, raises questions about the genetic background of the individuals rated as resistant or susceptible. One possibility is, that by chance none of the polymorphisms detected in this study was actually linked to QTL-2. Another possibility is, that one or more of the individuals of the susceptible bulk (rating = 9, plants are dead) harbored QTL-2, but were nevertheless killed. In this case, polymorphism linked to QTL-2 would not be detected with BSA. However, DNA markers closely linked to this locus will be important for molecular breeding for Ascochyta resistance, since this complex trait could be resolved into individual genetic components, which can be dealt with the efficacy of single genic traits (Tanksley, 1993).

The four STMS markers GA2, GAA47, GA24 and STMS 11, linked at maximum distances of about 20 to 27 cM to the resistance locus, are interesting for further research. STMS are the most informative markers available for chickpea (Hüttel et al., 1999; Winter et al., 1999). Therefore, at least one of the three markers will also identify polymorphisms in intra-species crosses segregating for resistances against the different pathotypes of the fungus, and should allow to determine if the same or another genomic region harbors major resistance genes against the other pathotypes. Unfortunately, we failed to generate a polymorphic, single-copy, codominant marker from OPS06-1. Such

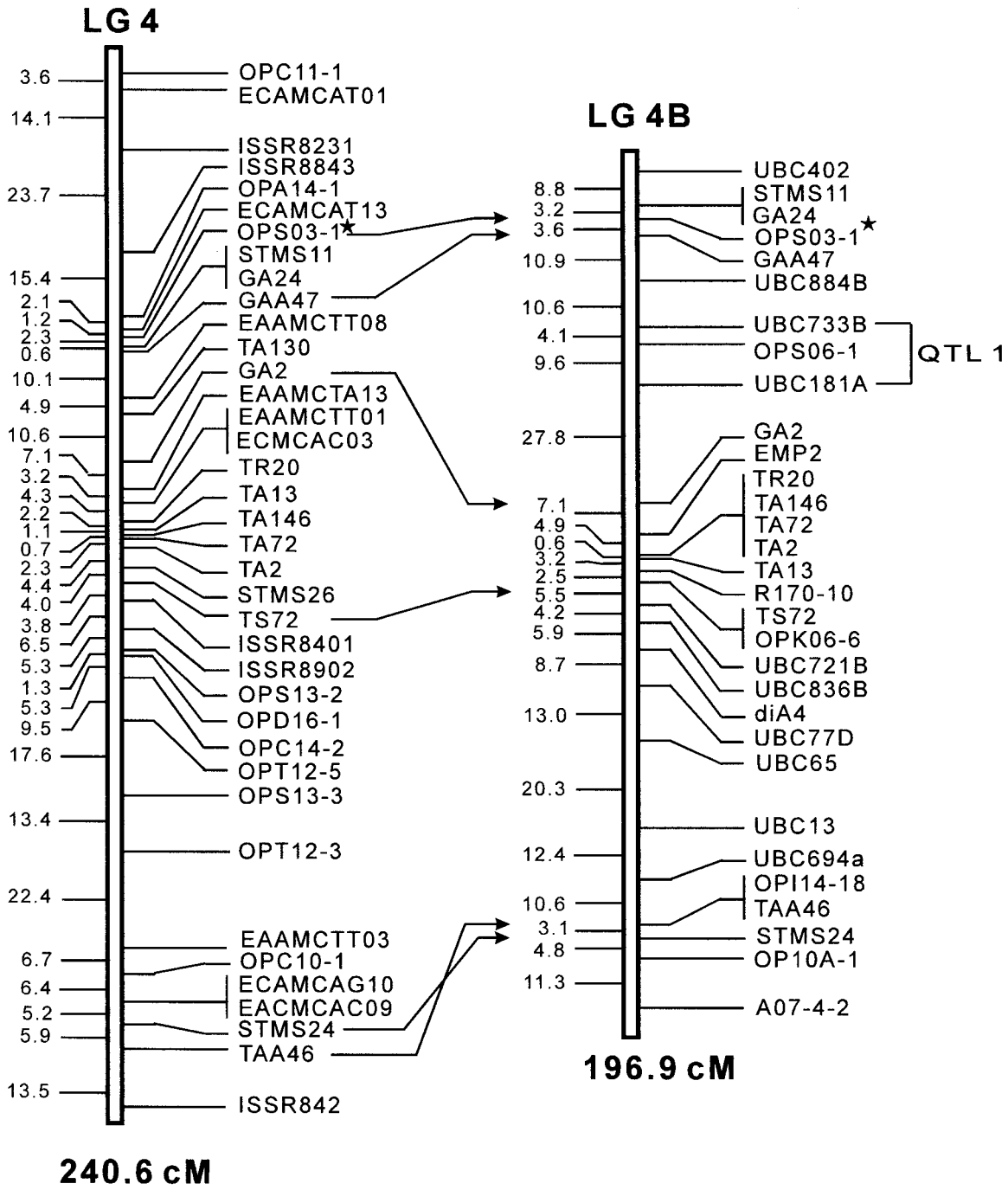

Figure 2. Comparison of marker orders on linkage group 4 in WC1 (LG4) and WC2 (LG4B). Markers on LG4 were described by Winter et al. (2000). Markers starting with UBC (*U*niversity of *B*ritish *C*olumbia) followed by a number are derived from PCR with random anchored or unanchored microsatellite-containing primers and are described by Santra et al. (2000). Markers starting with STMS, GA, GAA, TA, TAA, TR or TS are STMS markers described by Hüttel et al. (1999), Winter et al. (1999) and Winter et al. (2000), respectively. Markers starting with OP (*Op*eron) are produced by DAF with random 10mer oligonucleotides. Marker OPS03-1, which could be used for mapping in both populations, is marked by an asterisk. The number on the left of each LG indicates the distance between neighboring markers in cM.

a closely linked codominant marker could improve the efficiency of marker-assisted selection considerably. Moreover, the SCAR primers developed from such marker locus amplify a clear-cut unique band, and will identify already established bacterial artificial chromosome (BAC) clones carrying the locus. These may be searched for microsatellites which in turn may be used to generate STMS markers.

Another aim of the present study was to evaluate the marker order in linkage group 4 of the genetic maps of Winter et al. (1999, 2000). Our results confirm the published marker order, though some minor inconsistencies exist, e.g. a few tightly linked markers changed places relative to the other markers. These inconsistencies may derive from differences in recombination frequencies in distinct genomic regions between the two populations. These are already visible as large differences in map distances between distantly located markers and the lack of recombination between markers in WC2 tightly linked in WC1. Similar observations of variable distances between markers in different segregating chickpea populations were already reported by Kazan et al. (1993) and Simon and Muehlbauer (1997). These discrepancies can be explained in that recombination frequencies for specific regions may change from one F1 to another even in populations derived from crosses of the same parental lines. Therefore, fluctuations in map distances are not surprising. However, a realistic marker order will have to be determined in another, preferably intra-specific population. Marker-assisted pyramiding of resistance genes could eventually lead to durable resistance. However, this approach requires the tagging of resistance genes by closely linked markers. One such marker is now available.

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